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Potent and Specific Inhibition of Glutathione Synthesis by Buthionine Sulfoximine (*S*-*n*-Butyl Homocysteine Sulfoximine)*

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Buthionine sulfoximine (*S*-*n*-butyl homocysteine sulfoximine), the most potent of a series of analogs of methionine sulfoximine thus far studied (Griffith, O. W., Anderson, M. E., and Meister, A. (1979) *J. Biol. Chem.* 254, 1205-1210), inhibited γ -glutamylcysteine synthetase about 20 times more effectively than did prothionine sulfoximine and at least 100 times more effectively than methionine sulfoximine. The findings support the conclusion that the *S*-alkyl moiety of the sulfoximine binds at the enzyme site that normally binds the acceptor amino acid. Thus, the affinity of the enzyme for the *S*-ethyl, *S*-*n*-propyl, and *S*-*n*-butyl sulfoximines increases in a manner which is parallel to those of the corresponding isosteric acceptor amino acid substrates, i.e. glycine, alanine, and α -aminobutyrate. Buthionine sulfoximine did not inhibit glutamine synthetase detectably, nor did it produce convulsions when injected into mice. Injection of buthionine sulfoximine into mice decreased the level of glutathione in the kidney to a greater extent (<20% of the control level) than found previously after giving prothionine sulfoximine. α -Methyl buthionine sulfoximine was also prepared and found to be almost as effective as buthionine sulfoximine; this compound would not be expected to undergo substantial degradative metabolism. Buthionine sulfoximine and α -methyl buthionine sulfoximine may be useful agents for inhibition of glutathione synthesis in various experimental systems.

Previous studies in this laboratory showed that prothionine sulfoximine (*S*-*n*-propyl homocysteine sulfoximine) is a very effective inhibitor of γ -glutamylcysteine synthetase, and that this sulfoximine does not significantly perturb the activity of glutamine synthetase (1). This work was part of a study in which we have prepared analogs of methionine sulfoximine (which inhibits both glutamine synthetase and γ -glutamylcysteine synthetase) that selectively inhibit each of these enzymes. Thus, α -ethylmethionine sulfoximine was found to inhibit glutamine synthetase, but not γ -glutamylcysteine synthetase (2). By substituting bulkier moieties in place of the methyl group of methionine sulfoximine, we obtained molecules that exhibit less affinity for glutamine synthetase and more affinity for γ -glutamylcysteine synthetase. On theoretical grounds (1) it would be expected that substitution of an *n*-butyl moiety for the methyl group of methionine sulfoximine would lead to an excellent inhibitor of γ -glutamylcysteine

synthetase since there is evidence that the *S*-alkyl moieties of these sulfoximines bind to the region of the active center of the enzyme that binds the acceptor amino acid (cysteine, α -aminobutyrate). Although we had intended in our previous work (1) to prepare buthionine sulfoximine (*S*-*n*-butyl homocysteine sulfoximine), this plan had to be abandoned because it was difficult in our laboratory to handle the amounts of mercaptan required. This difficulty has now been overcome by construction of an effective ventilation system. In addition, we have synthesized buthionine sulfoximine by an alternative pathway that does not involve the use of butyl mercaptan. We report here that buthionine sulfoximine is highly active as an inhibitor of γ -glutamylcysteine synthetase *in vitro* and *in vivo*.

EXPERIMENTAL PROCEDURES

Materials—L-Glutamate, L-methionine-*SR*-sulfoximine, ATP, and phosphoenolpyruvate were obtained from Sigma. L- α -Aminobutyrate was obtained from Vega-Fox. L-Ethionine-*SR*-sulfoximine and DL-prothionine-*SR*-sulfoximine were prepared as described (1). Rat kidney γ -glutamylcysteine synthetase was purified from Holtzman rats (3).

DL-Buthionine was prepared as follows. The entire procedure was carried out in a very efficient fume hood. A mixture of 56.1 g of acrolein and 90.2 g of 1-butanethiol was magnetically stirred in an ice-salt bath in a flask fitted with a nitrogen inlet, thermometer, and an efficient reflux condenser. While the apparatus was being flushed with nitrogen, the mixture was cooled to 0°C, and 2 μ l of piperidine was added. After several minutes, a moderately strong exothermic reaction occurred and the temperature rose to 40–50°C. The solution was allowed to cool to 0°C and an additional 9 g of 1-butanethiol was added; the temperature of the mixture was allowed to rise to about 25°C. After standing for 2 h, the mixture was warmed to 70°C for 30 min and then allowed to cool to room temperature. The crude product was distilled using a water aspirator to yield a forerun of 1-butanethiol, followed by 129 g (88%) of 3-buthylthiopropionaldehyde; b.p.₁₅ 92–95°C. The purified aldehyde was converted to the hydantoin as follows. Ammonium carbonate (140 g) was dissolved in 500 ml of water at 60°C. To this solution was added 500 ml of ethanol, 24.5 g of sodium cyanide, and 48.3 g of 3-buthylthiopropionaldehyde. The mixture was stirred at 60°C throughout the reaction. A wide bore condenser was required to avoid clogging by the sublimed ammonium carbonate. After 24 h, the brown mixture was cooled to room temperature and the bulk of the ammonium carbonate and ethanol was removed by distillation at slightly reduced pressure. When the volume had been reduced by about 50%, the solution was cooled in ice, poured into a large beaker, and cautiously acidified (pH less than 3) by adding concentrated hydrochloric acid. 5-(3-Buthylthiopropyl)hydantoin precipitated and was collected by filtration and washed with water. The crude damp hydantoin was dissolved in 500 ml of 2 *M* sodium hydroxide and refluxed for 18 h. The residual ammonia and part of the solvent were removed by rotary evaporation under reduced pressure and the remaining solution was neutralized by addition of hydrochloric acid. Buthionine precipitated; the product was crystallized from water. The yield of DL-buthionine was 60 to 75%, based on the aldehyde.

The synthesis of α -methylbuthionine was carried out by an analogous procedure starting with 2-buthylthioethylmethylketone (b.p. 65–

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70°C). This compound was obtained by a procedure similar to that described above in 78% yield from 1-butanethiol and methylvinylketone; 300 μ l of piperidine catalyst was required for a reaction on a 1 mol scale. The ketone was converted to the amino acid as described above for the preparation of buthionine.

DL-Buthionine was also prepared by a slight modification of the method of Kolenbrander (4); this procedure does not require use of butanethiol. A 2-liter 3-neck round bottom flask containing 1 liter of methanol was fitted with a condenser and a nitrogen inlet. Using magnetic stirring and a slow flow of nitrogen, 20 g (1 mol) of freshly cut sodium was added in small (about 1 g) pieces. After the sodium had completely reacted and the solution had cooled to room temperature, 68 g of DL-homocysteine thiolactone hydrochloride (0.43 mol) was added, and the solution was stirred under nitrogen for about 30 min. To this solution (which contained insoluble sodium chloride) was added 87 g of 1-iodobutane (0.47 mol) and the reaction was stirred at room temperature overnight. The mixture was then filtered and the filtrate was reduced by rotary evaporation under reduced pressure to about 200 to 300 ml. To the concentrated solution was added 500 ml of 4 M sodium hydroxide and this mixture was refluxed for 6 h. After filtration and neutralization with concentrated hydrochloric acid, about 75 g (91%) of DL-buthionine was obtained.

DL-Buthionine and α -methyl-DL-buthionine were converted to their respective sulfoximines by a modification of the procedure of Bentley *et al.* (5). The methionine analog (80 mmol) was vigorously stirred at 55°C in a mixture of 60 ml of chloroform and 17 ml of concentrated sulfuric acid. A total of 140 mmol of sodium azide was added in small portions over a period of 12 h. The mixture was then allowed to cool to room temperature. The mixture was treated with cold water (400 ml), and the aqueous phase was removed. The chloroform layer was washed with several portions of water, and the combined aqueous solution was then loaded on a column of Dowex 50-H⁺. The column was washed with water, and the amino acid was then eluted with 8 M ammonium hydroxide. Flash evaporation of the eluate and crystallization of the residue from aqueous ethanol gave the sulfoximines in 50 to 75% yield.

Buthionine (m.p., 254–255°C (decomposed) (corrected); literature 250–252°C (4)).



Calculated: C 50.23 H 8.96 N 7.32
Found: C 50.67 H 8.66 N 7.25

α -Methyl-DL-buthionine (m.p., 268–274°C (discolors), 278–280°C (decomposed)).



Calculated: C 52.63 H 9.33 N 6.85
Found: C 53.21 H 9.25 N 6.80

DL-Buthionine-SR-sulfoximine (m.p., 214–215.5°C (decomposed) (corrected); literature m.p., 214°C (6)).



Calculated: C 43.22 H 8.16 N 12.60
Found: C 43.26 H 8.12 N 12.50

α -Methyl-DL-buthionine-SR-sulfoximine (m.p., 199–201°C (decomposed) (corrected)).



Calculated: C 45.74 H 8.53 N 11.85
Found: C 45.80 H 8.58 N 11.70

DL-Buthionine, α -methyl-DL-buthionine, DL-buthionine-SR-sulfoximine, and α -methyl-DL-buthionine-SR-sulfoximine all elute as single peaks on a Durrum model 500 amino acid analyzer. Using the standard hydrolysis procedure, the elution times are 68 min, 35 s, 62 min, 4 s, 53 min, 39 s, and 60 min, 39 s, respectively. Under similar conditions methionine, leucine, and phenylalanine elute at 46 min, 58 s, 60 min, 20 s, and 57 min, 45 s, respectively.

Methods—Enzymic assays were carried out at 30°C. The experimental details are given in Fig. 1 and Table I.

RESULTS

Fig. 1 summarizes data on the inhibition of γ -glutamylcysteine synthetase by buthionine sulfoximine, prothionine sulf-

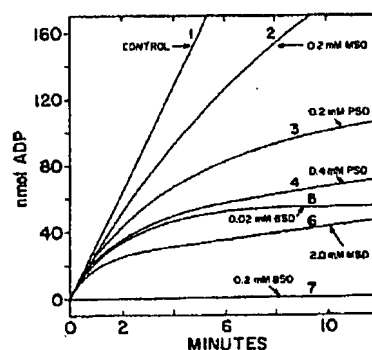


FIG. 1. Time course of γ -glutamylcysteine synthesis in the presence and absence of analogs of methionine sulfoximine. The reaction mixtures contained (final volume, 1.0 ml) 150 mM Tris-HCl buffer (pH 8.2), 76 mM KCl, 10 mM ATP, 5 mM phosphoenolpyruvate, 25 mM MgCl₂, 6 mM sodium L-glutamate, 10 mM L- α -aminobutyrate, 0.2 mM NADH, 0.2 mM EDTA, 10 IU of pyruvate kinase, 11 IU of lactate dehydrogenase, and 1.92 units of γ -glutamylcysteine synthetase. Curve 1, control; time course of the reaction without inhibitor. In the other experiments, inhibitors were present as follows: Curve 2, 0.2 mM L-methionine-SR-sulfoximine (MSO); Curve 3, 0.2 mM DL-prothionine-SR-sulfoximine (PSO); Curve 4, 0.4 mM DL-prothionine-SR-sulfoximine; Curve 5, 0.02 mM DL-buthionine-SR-sulfoximine (BSO); Curve 6, 2.0 mM L-methionine-SR-sulfoximine; Curve 7, 0.2 mM DL-buthionine-SR-sulfoximine.

oximine, and methionine sulfoximine. In these studies the reaction was initiated by addition of the enzyme to a buffered solution containing the inhibitor and the substrates. The rate of reaction falls off in the experiments shown in Curves 2 to 7 due to enzymic inhibition associated with phosphorylation of the sulfoximines and tight binding of the sulfoximine phosphate derivatives to the enzyme (1, 7–10). In agreement with previous findings (1), 0.2 mM prothionine sulfoximine (Curve 3) inhibited activity to a much greater extent than did 0.2 mM methionine sulfoximine (Curve 2). However, 0.02 mM buthionine sulfoximine (Curve 5) was significantly more inhibitory than 0.4 mM prothionine sulfoximine (Curve 4). Under these conditions, 0.2 mM buthionine sulfoximine (Curve 7) produced virtually complete inhibition. It may be estimated from these findings (see also Table I) that buthionine sulfoximine, which produced 52% inhibition under these conditions at a concentration of 1 μ M, is (a) at least 100 times more inhibitory than methionine sulfoximine (which produced 52% inhibition at a concentration of 100 μ M) and (b) about 20 times more inhibitory than prothionine sulfoximine. It is striking that 0.02 mM buthionine sulfoximine (Curve 5) led to complete inhibition after 10 min, whereas 2 mM methionine sulfoximine (Curve 6) did not inhibit completely. It is of note that α -methyl buthionine sulfoximine, although a highly potent inhibitor, is somewhat less effective than buthionine sulfoximine.

Buthionine sulfoximine (0.5 mM) did not inhibit glutamine synthetase when preincubated with the enzyme, ATP, and Mg²⁺ under the conditions previously described (1). Injection of buthionine sulfoximine into mice at a dose of 32 mmol/kg did not produce convulsions or detectable behavioral change over a period of observation of 7 days. The kidney levels of glutathione found in mice 1.5 h after injection of buthionine sulfoximine (4 mmol/kg) were depressed to less than 20% of the control levels; less depression was observed after injection of prothionine sulfoximine (1). In addition, mice given buthionine sulfoximine had depressed renal glutathione levels after 12 h; after an equivalent dose of prothionine sulfoximine, the renal glutathione levels returned to normal after about 10 h (1).

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TABLE I
Inhibition of γ -glutamylcysteine synthetase by analogs of methionine sulfoximine

Sulfoximine	Inhibition of γ -glutamylcysteine synthetase ^a		Isosteric acceptor amino acid ^b	K_m of amino acid ^c
	10 μ M ^d	100 μ M ^d		
	% inhibition			mM
L-Methionine-(SR)-sulfoximine	8	62		
L-Ethionine-(SR)-sulfoximine	3	35	Glycine	>250
DL-Prothionine-(SR)-sulfoximine	26	96	L-Alanine	75
DL-Buthionine-(SR)-sulfoximine	100	100	L- α -Aminobutyrate	3.3
α -Methyl-DL-buthionine-(SR)-sulfoximine	52 ^e	100	L- α -Aminobutyrate	3.3
	9 ^f			

^a The reaction mixtures (final volume, 1.0 ml) contained 150 mM Tris-HCl buffer (pH 8.0), 100 mM KCl, 5 mM sodium L-glutamate, 10 mM L- α -aminobutyrate, 10 mM ATP, 5 mM phosphoenolpyruvate, 20 mM MgCl₂, 0.2 mM EDTA, 0.2 mM NADH, 12 IU of pyruvate kinase, 10 IU of lactate dehydrogenase, and sulfoximine as indicated. The reaction was initiated by addition of 0.58 unit of γ -glutamylcysteine synthetase, and the oxidation of NADH (equivalent to ADP formation) was monitored at 340 nm. The extent of the reaction at 10 min was compared to a sulfoximine-free control to determine the per cent inhibition.

^b The sense in which α -aminobutyrate and the S-alkyl group of buthionine sulfoximine are isosteric is shown in Fig. 2 (see also Fig. 5 of Ref. 1).

^c K_m values were determined from double reciprocal plots of data from rate measurements in which the concentrations of ATP and glutamate were saturating. The reaction mixtures (final volume, 1.0 ml) contained 150 mM Tris-HCl (pH 8.2), 100 mM KCl, 10 mM L-glutamate, 10 mM ATP, 5 mM phosphoenolpyruvate, 20 mM MgCl₂, 0.2 mM EDTA, 0.2 mM NADH, 12 IU of pyruvate kinase, 10 IU of lactate dehydrogenase, and varying amounts of acceptor amino acid. The reaction was initiated by addition of 0.5 to 2 units of γ -glutamylcysteine synthetase and the formation of ADP was determined as described above.

^d Concentration of the L-S isomer of the sulfoximine present in the incubation mixture. The calculation of isomer concentration was based on the assumption that the possible diastereomers were present in equimolar amounts.

^e Per cent inhibition observed with a sulfoximine concentration of 1 μ M.

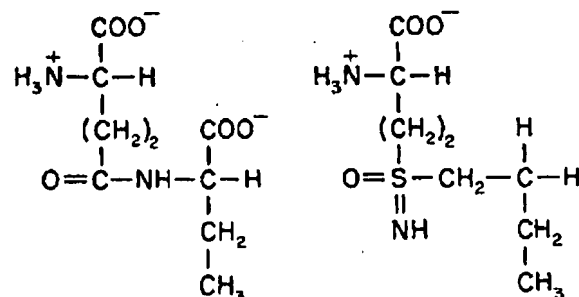


FIG. 2. Structures of γ -glutamyl- α -aminobutyrate (left) and buthionine sulfoximine (right).

DISCUSSION

Buthionine sulfoximine and α -methylbuthionine sulfoximine are the most potent sulfoximine inhibitors of γ -glutamylcysteine synthetase in the large series of sulfoximines that we have thus far examined. The data indicate that glutathione biosynthesis may be inhibited by concentrations of buthionine sulfoximine that are only 1% of that required of methionine sulfoximine and only 5% of that required of prothionine sulfoximine. Whereas prothionine sulfoximine produced only very slight inhibition of glutamine synthetase (1), buthionine sulfoximine did not inhibit glutamine synthetase detectably. Injection of prothionine sulfoximine (32 mmol/kg) produced only mild convulsions (after mechanical stimulation of the mice) (1), whereas injection of the same dose of buthionine sulfoximine was not followed by any abnormal behavior. These observations suggest that buthionine sulfoximine may be somewhat more useful as a selective inhibitor of glutathione synthesis in *in vivo* systems than prothionine sulfoximine. In certain studies the use of α -methyl buthionine sulfoximine may be desirable since this compound would be expected to be less susceptible to degradative metabolism; thus, it would not be a substrate for oxidative deamination or transamination reactions.

The data reported here give additional support to the interpretations expressed previously (1) about the active site of γ -glutamylcysteine synthetase, according to which the S-alkyl moiety of the sulfoximine was postulated to bind at the enzyme site that normally binds the acceptor amino acid. As indicated in Table I, the affinity of the enzyme for the S-ethyl, S-n-propyl, and S-n-butyl sulfoximines increases in a manner which is parallel to those of the corresponding isosteric acceptor amino acid substrates, i.e. glycine, alanine, and α -aminobutyrate. The explanation for the finding that methionine sulfoximine is more inhibitory than ethionine sulfoximine is not yet entirely clear. Methionine sulfoximine would not be expected to interact appreciably with the site for the binding of acceptor amino acid, and this may allow it to align more precisely with the glutamate binding site. It is also possible that the ethyl moiety of ethionine sulfoximine is hindered in binding by the same structural features of the enzyme that restrict the binding of isoprothionine sulfoximine, which is a poor inhibitor (1).

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